Remarks

I. Status of the Claims

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendments, claims 1-13, 17, 19-32 and 45-70 are pending in the present application, with claims 1 and 45 being the independent claims. Claims 1 and 45 are sought to be amended. Support for the amendments to claims 1 and 45 may be found in the specification as filed, *e.g.*, at page 10, lines 20-25, lines 28-29 and lines 32-34; page 11, lines 1-2; page 16, lines 8-19 and Examples 1 and 2. These amendments are sought to place the claims into condition for allowance or for consideration on appeal, and introduce no new matter. Entry and consideration of these amendments are respectfully requested. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

II. Summary of the Office Action

In the Office Action dated June 26, 2009, the Examiner has made four rejections of the claims. Applicants respectfully offer the following remarks concerning each of these elements of the Office Action.

III. The Rejection Under 35 U.S.C. § 112, First Paragraph is Traversed

At pages 2-3 of the Office Action, the Examiner has rejected claims 1-13, 17, 19-32 and 45-70 for allegedly failing to comply with the written description requirement. Applicants respectfully traverse.

The Examiner alleges that the phrases "wherein said culture of pluripotent cells has a concentration of about 0.5 x 10^6 to 5 x 10^6 cells/ml" and "wherein said culture of pluripotent cells has a concentration of about 0.1 x 10^6 to 1 x 10^6 cells/ml" are not supported by the specification. Applicants respectfully disagree. As discussed in the interview with Examiner Chen on September 15, 2009, support for these phrases can be clearly found in the specification as filed at page 10, lines 20-21 and lines 32-33 and page 11, lines 22-27. Specifically, the specification discloses two protocols for generating embryoid bodies. The first protocol is used when the initial cell density is from 1 to 5 X 10^6. The second protocol is used when the initial cell density is 0.1 to 0.5 X 10^6. When the initial cell density is between 0.5 to 1 X 10^6, either protocol 1 or protocol 2 can be used. *See* specification at page 11, lines 22-27 and page 10, lines 20-21 and lines 32-33. Thus, protocol 1 which is represented by claim 1 can be used when the initial cell density is 0.5 X 10^6 to 5 X 10^6, while protocol 2 which is represented by claim 45 can be used when the initial cell density is 0.1 X 10^6 to 1 X 10^6. Accordingly, the claims as currently presented are fully described in the present specification as filed. Thus, as acknowledged by Examiner Chen in the Interview Summary dated September 15, 2009, Applicants respectfully submit that the rejection of claims 1-13, 17, 19-32 and 45-70 for lack of written description has been overcome. Therefore, reconsideration and withdrawal are respectfully requested.

IV. The Rejections Under 35 U.S.C. § 103(a) are Traversed

A. Claims 1-3, 7-13, 17, 31, 32, 45-47, 51-54, 67 and 68

At pages 4-9 of the Office Action, the Examiner has rejected claims 1-3, 7-13, 17, 31, 32, 45-47, 51-54, 67 and 68 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson *et al.*, 2003 (U.S. Patent No. 6,602,711 B1; hereinafter "Thomson") or Dang *et al.*, June 20, 2003 (U.S. 2003/0119107 A1; hereinafter "Dang") each in view of Yan *et al.*, February 2003 (U.S. 2003/0027331; hereinafter "Yan") and Kehat *et al.*, 2001 (The Journal of Clinical Investigation, Vol. 108, No. 3, p. 407-414; hereinafter "Kehat"). Applicants respectfully traverse this rejection with regard to the currently amended claims.

The factors to be considered under 35 U.S.C. § 103(a), are the scope and content of the prior art; the differences between the prior art and the claims at issue; and the level of ordinary skill in the pertinent art. *See Graham v. John Deere*, 86 S.Ct. 684 (1966) and MPEP §2141. This analysis has been the standard for 40 years, and remains the law today. *See KSR International Co v. Teleflex Inc.*, 127 S.Ct. 1727 (2007). The Office has recently published Examination Guidelines to aid Examiners in formulating obviousness rejections. *See* MPEP § 2141 (hereinafter "the Examination Guidelines"). Seven rationales are suggested by which obviousness may be found, *e.g.*, by combining elements in the art or substituting one known element for another. As a common thread through all the rationales, the Examiner must establish on the record that a person of ordinary skill in the art would have recognized that the results of the combination or substitution were *predictable*. *Id.*

The Examiner has not met the burden of establishing a *prima facie* case of obviousness based on the Examination Guidelines. Specifically, the Examiner has not

established that the ordinary artisan reading Thomson or Dang and Yan and Kehat in combination would arrive at the presently claimed method that uses the rocking of a container containing a liquid single cell suspension culture of a certain amount and concentration of pluripotent cells to generate a high volume and density of embryoid bodies (EBs).

First, in order to clarify the invention and further prosecution and not in acquiescence to the Examiner's rejection, Applicants have amended independent claims 1 and 45 to recite "rocking a container containing a liquid single cell suspension culture of pluripotent cells" instead of "agitating a liquid single cell suspension culture of pluripotent cells." Support for these amendments can be found in the specification as filed at page at page 10, lines 20-25, lines 28-29 and lines 32-34; page 11, lines 1-2; page 16, lines 8-19 and Examples 1 and 2.

The present invention provides for the generation of large amounts of high quality embryoid bodies (EBs). The invention is based on the observation that agitation such as rocking of a certain amount and concentration of a single cell suspension of ES cells is superior to the other conventional methods of embryoid body formation. *See* specification at page 9, lines 8-14. As discussed in the specification, the agitation (*e.g.*, rocking) of a single cell suspension of pluripotent cells allows for the generation of EBs in high density which has no negative influence on the differentiation capacity towards different cell types such as cardiomyocytes, neurons, endothelial cells and liver cells. *See* specification at page 9, lines 14-17. For example, the ES cells are less exposed to shear stress than the conventional spinner flasks or stirring cultures, thus the ability of the cells to differentiate in an appropriate manner is not negatively influenced. *See* specification at page 10, lines 7-9. Accordingly, the method of the present invention is able to generate a large amount and high density of embryoid bodies compared to the conventional methods. *See* specification at page 9, lines 8-

10 and page 17, lines 1-4. In contrast, the art cited by the Examiner would not have provided a reasonable expectation of success in obtaining the presently claimed methods because the mere substitution or combination of elements from the four references cited by the Examiner would not have resulted in a predicable method of producing embryoid bodies from a single cell suspension of pluripotent cells, as presently claimed.

First, Thomson teaches a method for producing embryoid bodies from embryonic stem cells in the form of "**clumps**". Thus, contrary to the claimed invention, Thomson does not teach a method of producing embryoid bodies from a single cell suspension, *i.e.*, from single individual cells, but from cell aggregates, *i.e.*, clumps. In particular, Thomson describes the generation of ES cell aggregates by partially dissociating overgrown or piled cultures of ES cells into clumps, which are then cultured in suspension to induce further differentiation. Thomson's method of embryoid body formation does not teach treatment of the cells in order to obtain a single cell suspension. In fact, Thomson teaches that primate ES cells die rapidly when dispersed to single cells if attachment is prevented (*e.g.*, by rocking). *See* Thomson at col. 2, lines 30-32. Thus, one of ordinary skill in the art would not have expected that a single cell suspension of primate ES cells could be used to generate embryoid bodies with a reasonable expectation of success using the method of the present invention.

In addition, while Thomson does suggest incubation on a rocking table or a non-adherent plastic dish, clearly, the method described in Thomson uses the rocking table not for generation of aggregates themselves, but rather only to prevent the already existing "clumps" of cells from sticking to the surface during culture. In addition, the method of Thomson only suggests the incubation of "clumps" on the rocking table, not a single cell suspension of

pluripotent cells. In contrast, as illustrated in Example 1, the pluripotent stem cells subjected to the method of the present invention are present as a single cell suspension which is rocked until formation of the cell aggregates and EBs. *See* specification at page 42, lines 15-26. Thus, based on the disclosure of Thomson, one of ordinary skill in the art would not have predicted that rocking of a single cell suspension of pluripotent cells would generate a high volume and density of EBs.

Dang is directed toward the improvement of stirred bioreactors and differentiation of cells in stirred cultures. Indeed, as discussed below, Dang does not come even remotely close to the present invention. The description of Dang as a whole makes it abundantly clear that no method, other than stirring, is to be used for the generation of EBs in order to achieve the desired effect, *i.e.*, prevention of aggregation between separate EBs. Hence, as confirmed by the examples, stirred cultures are always used in the method of Dang. *See* Dang, *e.g.*, at paragraph [0113] and Fig. 2.

The fact that the teaching of Dang concerning the preparation of EBs is confined to stirred cultures is further corroborated by the inventors and applicants of Dang. For example, in the reply to a Communication pursuant to Article 96(2) EPC (Exhibit 1) issued by the European Patent Office on January 19, 2005 in European patent application No. 02 745 012.1, which is the corresponding European application to the Dang reference, the Applicants state at page 2, first full paragraph that "the encapsulation technology of the present invention solves the problem, enabling the use of stirred cultures without EB interaction,...." Further, at page 3 of the reply, emphasis is put on the use of stirred suspension cultures and stirred bioreactors, respectively. In support of its contention, the reply is accompanied by two publications from the inventors of the Dang reference, which

emphasize the use of stirred suspension by reactors. *See* Dang *et al.*, *Stem Cells 22*: 275-282 (2004) in the abstract (Exhibit 2) and Bauwens *et al.*, *Biotechnology and Bioengineering 90*: 452-461 (2005) (Exhibit 3). In this context, the Examiner is kindly referred to the abstract of Exhibit 2 (left column, third sentence) which states that "[a]ggregation between EBs (agglomeration), however, inhibits cell growth and differentiation in stirred or high-cell-density static cultures" and the last sentence of the abstract referring to the use of stirred-suspension bioreactors. As can be inferred from these statements, according to the inventors of Dang only two options for the aggregation of EBs existed at the time, *i.e.*, (1) static cultures such as hanging drop and methylcellulose cultures and (2) stirred cultures which is consistent with the teaching of Dang. *See* Dang at paragraphs [0008] to [0013].

As discussed in the specification, the method of the present invention is superior to the stirring method (*e.g.*, spinner flask technology) for generating embryoid bodies. Specifically, as indicated above, compared to cultures in spinner flasks, in the agitation method (*e.g.*, rocking) the ES cells are much less exposed to shear stress, whereby the capability of the cells to differentiate in an appropriate manner is not negatively influenced. *See* specification at page 10, lines 7-9. In addition, the specification points out that in previous methods for the production of embryoid bodies the yield of embryoid bodies was in the range of 50/ml. However, using the present method, one of ordinary skill in the art could obtain embryoid bodies generally in the range of 500/ml. *See* specification at page 17, lines 1-5. Thus, these results confirm that the agitation method of the present invention is superior to the stirring method for generating embryoid bodies.

This conclusion is further supported by the data presented in the Declaration Under 37 C.F.R. § 1.132 of Dr. Silke Schwengberg (hereinafter "the Schwengberg Declaration"). As

discussed in the Schwengberg Declaration, the agitation method of the present invention was compared to the stirring method found in the art. *See* the Schwengberg Declaration at paragraphs 9-12. The agitation method produced a higher (i) yield of EB per ml and (ii) yield of differentiated cells (*e.g.*, cardiac cells) per ES cell originally seeded into the differentiation culture compared to the stirring method. *See* the Schwengberg Declaration at paragraphs 9-13.

Thus, Dang does not teach or suggest that rocking of a single cell suspension culture of pluripotent cells would produce a high volume and density of embryoid bodies. Dang only suggests methods of improving the already known stirring method which as indicated above is inferior to the method of the present invention. Thus, based on the disclosure of Dang, one of ordinary skill in the art would not have predicted that rocking of a single cell suspension of pluripotent cells would generate a high volume and density of EBs.

The deficiencies of Thomson and Dang are not cured by the disclosure of Yan and Kehat. The Examiner asserts that is would have been *prima facie* obvious to grow pluripotent cells in single cell suspension because Yan teaches growing homozygous stem (HS) cells, which are pluripotent cells, in single cell suspension to form embryoid bodies. However, while Yan teaches using a single cell suspension to generate embryoid bodies, Yan does not suggest or teach a method for generating embryoid bodies by rocking a single cell suspension of pluripotent cells. The method used in Yan to generate the embryoid bodies is a static culture. Nowhere in Yan is it taught or suggested that a single cell suspension culture should be rocked in order to produce a high volume and density of EBs. Accordingly, one of ordinary skill in the art would not have predicted based on Yan that rocking of a single cell suspension of pluripotent cells would generate a high volume and density of EBs.

Kehat, like Thomson, teaches using **clumps** of ES to generate EBs, not a **single cell** suspension, as claimed. *See* Kehat at page 408, the paragraph bridging left and right column. Furthermore, like Yan, the culture taught in Kehat is a static culture. *See* Fig. 1 of Kehat at page 408 and the figure legend. Nowhere in Kehat is it taught or suggested that a single cell suspension culture should be rocked in order to produce a high volume and density of EBs. Accordingly, one of ordinary skill in the art would not have predicted based on Kehat that rocking of a single cell suspension of pluripotent cells would generate a high volume and density of EBs.

None of the references cited by the Examiner teach a method of producing embryoid bodies from pluripotent cells by rocking a single cell suspension with a concentration of 0.5x 10^6 to 5x 10^5 cells/ml or 0.1x 10^6 to 1x 10^6 cells/ml. The mere substitution or combination of elements from the combination of references cited by the Examiner would not have resulted in a predicable method of producing embryoid bodies from pluripotent cells, as presently claimed. Thus, for at least the foregoing reasons, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness as set out in the Examination Guidelines and respectfully request that this rejection under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

B. Claims 1-6 and 45-50

At pages 9-12 of the Office Action, the Examiner has rejected claims 1-6 and 45-50 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson or Dang each in view of Yan and Kehat and further in view of Dang *et al.*, *Biotechnol. Bioeng. 78*:442-453 (2002) ("hereinafter Dang *et al.*"). Applicants respectfully traverse this rejection.

As discussed above, the present invention provides for the generation of large amounts of high quality embryoid bodies (EBs). The invention is based on the observation that agitation such as rocking of a certain amount and concentration of a single cell suspension of ES cells is superior to the other conventional methods of embryoid body formation. *See* specification at page 9, lines 8-14. As discussed in the specification, the agitation (*e.g.*, rocking) of a single cell suspension of pluripotent cells allows for the generation of EBs in high density which has no negative influence on the differentiation capacity towards different cell types such as cardiomyocytes, neurons, endothelial cells and liver cells. *See* specification at page 9, lines 14-17. For example, the ES cells are less exposed to shear stress than the conventional spinner flasks or stirring cultures, thus the ability of the cells to differentiate in an appropriate manner is not negatively influenced. *See* specification at page 10, lines 7-9. Accordingly, the method of the present invention is able to generate a large amount and high density of embryoid bodies compared to the conventional methods. *See* specification at page 9, lines 8-10 and page 17, lines 1-4. In contrast, the art cited by the Examiner would not have provided a reasonable expectation of success in obtaining the presently claimed methods because the mere substitution or combination of elements from the five references cited by the Examiner would not have resulted in a predicable method of producing embryoid bodies from a single cell suspension of pluripotent cells, as presently claimed.

Thomson, Dang, Yan and Kehat have been discussed in detail above. Dang *et al.* discloses maintenance and differentiation of CCE murine embryonic stem cells using the conventional methods such as liquid suspension cultures, methycellulose cultures, hanging drop cultures, or stirring cultures. However, Dang *et al.* does not teach or suggest that a

single cell suspension culture could be rocked in order to generate EBs in large amounts and high density. Accordingly, one of ordinary skill in the art would not have predicted based on Dang *et al.* that rocking of a single cell suspension of pluripotent cells would generate a high volume and density of EBs.

In contrast to the cited references, the present invention provides for the production of embryoid bodies in large amounts and high density by rocking a certain amount and concentration of pluripotent cells. *See* specification at page 9, lines 6-14. None of the references cited by the Examiner teach a method of producing embryoid bodies from pluripotent cells by rocking a single cell suspension of a certain amount and concentration of pluripotent cells. Applicants respectfully assert that the ordinary artisan reading the combination of references could not have predictably arrived at the presently claimed invention of producing embryoid bodies (EBs) from pluripotent cells by rocking a container containing a liquid single cell suspension culture of pluripotent cells with a concentration of $0.5x\ 10^6$ to $5x\ 10^5$ cells/ml or $0.1x\ 10^6$ to $1x\ 10^6$ cells/ml to generate EBs. Therefore, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness as set out in the Examination Guidelines and respectfully request that this rejection be withdrawn.

C. Claims 1, 17, 19-30, 54-66, 69 and 70

At pages 12-14 of the Office Action, the Examiner has rejected claims 1, 17, 19-30, 54-66, 69 and 70 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Thomson in view of Yan and Kehat and further in view of Dang. Applicants respectfully traverse this rejection.

Thomson, Dang, Yan and Kehat have been discussed in detail above. As discussed above, none of the references cited by the Examiner teach a method of producing embryoid bodies from pluripotent cells by rocking a single cell suspension of a certain amount and concentration of pluripotent cells. Applicants respectfully assert that the ordinary artisan reading the combination of references could not have predictably arrived at the presently claimed invention of producing embryoid bodies (EBs) from pluripotent cells by rocking a container containing a liquid single cell suspension culture of pluripotent cells with a concentration of 0.5x 10^6 to 5x 10^5 cells/ml or 0.1x 10^6 to 1x 10^6 cells/ml to generate EBs. Therefore, Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness as set out in the Examination Guidelines and respectfully request that this rejection be withdrawn.

V. Statement of the Substance of Interview

Applicants submit the following Statement of the Substance of the Interview in accordance with MPEP § 713.04. Applicants thank Examiner Shin Lin Chen for his time in participating in a personal interview with Shannon A. Carroll and Elizabeth J. Haanes on September 15, 2009, to discuss the outstanding Office Action. During the interview, Applicants and the Examiner discussed the 35 U.S.C. §§ 112, first paragraph and 103(a) rejections.

VI. Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all

presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

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Exhibit 1

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The European Patent Office
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26 July 2005

Dear Sirs

European Patent Application No. 02745012.1-2401
ZANDSTRA, Peter W., et al
Our Ref : N.91034 SER/mr

This is in response to the Communication issued 19 January 2005 pursuant to Article 96(2) EPC. I am filing herewith a replacement set of claims to replace the claims as presently on file. I also enclose a copy of the previous claims showing the amendments being made at this time in manuscript.

Turning firstly to the amendments to the claims, the Examiner will see that claim 7 has been amended to specify that the bioprocess includes the step of differentiating the cells into a population of cardiomyocytes. Thus, it is quite clear that the claimed method does indeed include a further step. This step was effectively inherent in previous claim 7 and also find support for example in Example 5 on page 41.

Claim 20 has also been amended to clarify that it is a product claim directed to the culture system. We trust that the amendments to claims 7 and 20 take care of the clarity objections raised by the Examiner in section 4 of the latest Communication.

Turning in more detail to the Communication, the Applicant is pleased to see that the Examiner has acknowledged that the claims as filed meet the requirements of Article 123(2) EPC and are also considered to be novel over the cited prior art.

The Examiner suggests that the issue of aggregation of ES cells solved by the present invention was not a problem in the stirred cultures of reference D3, Wartenberg et al., Lab. Invest. 78:1301-14,

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1998. However we submit that this was a problem as evidenced by D3 itself, and the present application.

The data presented in the present application shows that interaction of embryoid bodies (EBs) is a real problem. Referring to Figure 3, the specification explains that EB formation efficiency decreases at all cell concentrations over time, see page 32, having the effect of reducing the rate of cell expansion (page 33) - which in turn impairs access to cell culture ingredients, thereby affecting differentiation (page 34). The encapsulation technology of the present invention solves the problem, enabling the use of stirred cultures without EB interaction, which provides an improved environment for controlling the differentiation process, see in particular page 37 of the present application.

The authors of D3 did not worry about an aggregation problem in their cultures, because they saw the EBs getting larger and retain a substantially spherical shape. In fact, their own data actually demonstrate that there was considerable EB interaction going on in their cultures.

In order to understand this, it is helpful to appreciate the "agglomeration" process. EBs are adapted to interact with each other, by virtue of cell adhesion molecules like E-cadherin. As described in the present application on page 33 (section (d)), the cells first adhere to each other by way of these surface molecules. However, the process does not stop there. Quantitative measurement of the diameter of the EB shows that the clustered cells *go on to fuse into a single large EB.*

This has been further illustrated in the subsequent paper by Dang et al., Stem Cells 22:275-82, 2004, a copy of which is attached to the confirmation copy of this letter by courier. As shown in Figure 1 on page 277, a pair of EBs adhered to each other will fuse over the course of 24 hours so that 95% of the two cell populations form a single larger sphere. The data in this paper demonstrate this conclusively, because the experiment was done with pairs of EBs in which each single EB was derived from different cell lines, *one of which expressed a fluorescent protein.* Complete fusion was verified by the demonstration that fluorescent and non-fluorescent cells merged into the resulting fused EB.

This explains data in the D3 reference that would otherwise be puzzling. Figure 2 of D3 shows EBs *changing in size* over the course of just 5 days of culture (Day 3 to Day 8). In Panel A, the EBs change from ~600 to ~1200 μm in diameter (an 8-fold increase in volume). In Panels B and C, they change from ~100 to almost ~350 μm (over 40-fold increase). It is inconceivable that the cells in the EBs could grow this much in 5 days. Obviously, the EBs in D3 are increasing their size at least in part by fusion of EBs together.

The aggregation and fusion problem of EBs in stirred culture, as illustrated inadvertently by the data in D3, is solved by the invention claimed in the present application. The Dang paper (*supra*) confirms that agarose-encapsulated EBs are considerably stabilized from aggregation and

agglomeration, see for example Figure 4, page 279 of this paper. The ability to use a stirred suspension culture without the fusion problem allows the user to rapidly implement changes in culture conditions through the differentiation process. This is shown by the formation of hematopoietic cells in an environment where oxygen tension is carefully controlled, see Figure 6, page 280. Another publication by Bauwens et al., Biotechnol. Bioeng. 90:452-61, 2005 a copy of which is also attached with the confirmation copy of this letter by courier further illustrates the use of the encapsulation technology in stirred bioreactors to form cardiomyocyte lineage cells.

Thus, this invention solves a genuine problem in the culture of EBs, thereby facilitating the production of therapeutically important cell types both in terms of the rate of growth, and improved homogeneity of the resultant cell population.

We trust that this takes care of all of the points raised in the latest Communication and that this application may now proceed to acceptance. Should there be any further minor points outstanding, the Examiner is invited to telephone the undersigned. Should the Examiner be minded to refuse this application without first issuing a further Communication, Oral Proceedings are hereby requested.

Please acknowledge receipt of this letter and its enclosures by date stamping and returning the enclosed acknowledgement copy.

Yours faithfully

Sarah Roques

SARAH E. ROQUES

Exhibit 2

STEM CELLS®
Rapid Communication

Controlled, Scalable Embryonic Stem Cell Differentiation Culture

STEPHEN M. DANG,[a] SHARON GERECHT-NIR,[b,c] JINNY CHEN,[a] JOSEPH ITSKOVITZ-ELDOR,[c,d] PETER W. ZANDSTRA[a]

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Key Words. *Stem cell culture · Encapsulation · Embryonic stem cells · Embryoid body · Scalable*

ABSTRACT

Embryonic stem (ES) cells are of significant interest as a renewable source of therapeutically useful cells. ES cell aggregation is important for both human and mouse embryoid body (EB) formation and the subsequent generation of ES cell derivatives. Aggregation between EBs (agglomeration), however, inhibits cell growth and differentiation in stirred or high-cell-density static cultures. We demonstrate that the agglomeration of two EBs is initiated by E-cadherin-mediated cell attachment and followed by active cell migration. We report the development of a technology capable of controlling cell-cell interactions in scalable culture by the mass encapsulation of ES cells in size-specified agarose capsules. When placed in stirred-suspension bioreactors, encapsulated ES cells can be used to produce scalable quantities of hematopoietic progenitor cells in a controlled environment. *Stem Cells 2004;22:275-282*

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells capable of extensive proliferation while maintaining their potential to differentiate into any cell type in the body [1, 2]. ES cells can therefore be considered a renewable source of useful cell types such as cardiomyocytes [3], insulin-secreting cells [4], dopaminergic neurons [5], and hematopoietic progenitors [6, 7]—cells that have proven difficult to expand in vitro. While ES-derived cells have tremendous potential in many experimental and therapeutic applications, their utility, and indeed investigations into the scope of their utility, is dependent on the availability of relevant cell quantities. This can be accomplished by increasing the scale of cell production and by optimizing culture conditions for the generation of target cells.

In an ideal scenario, differentiation of ES cells could be directed to a pure population of the desired cell type. For example, *Tropepe et al.* (2001) [8] described culture conditions that exclusively permit the formation of neural progenitor cells from mouse ES cells, albeit at very low cell frequency. In most cases, the knowledge to precisely control mouse or human ES cell fate decisions is lacking. Consequently, the most robust method for generating most differentiated cell types is through the embryoid body (EB) system where ES cells spontaneously differentiate as tissue-like spheroids in suspension culture. EB differentiation

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has been shown to recapitulate aspects of early embryogenesis, including the formation of a complex three-dimensional architecture wherein cell-cell and cell-matrix interactions are thought to support the development of the three embryonic germ layers and their derivatives [9, 10].

Presently, all human and most mouse ES cell lines require aggregation of multiple ES cells to efficiently initiate EB formation [10, 11]. Standard methods of EB formation include hanging drop, liquid suspension, and methylcellulose culture. These culture systems maintain a balance between allowing ES cell aggregation necessary for EB formation and preventing EB agglomeration for efficient cell growth and differentiation [11]. However, these culture systems are limited in their production capacity and are not easily amenable to process-control strategies. The ability to culture differentiating ES cells in stirred-suspension bioreactors, either alone or in combination with other technologies [12], would overcome many of the current culture limitations. Stirred-suspension bioreactors are readily scaled in size to match production needs. Stirring also homogenizes bulk media conditions and thus facilitates measurement and control. In addition to its cell-production-related role, the ability to control culture conditions would be useful for experimental investigations of extrinsic factors (such as glucose concentration, cytokine concentration, pH, and oxygen tension) and their effects on ES cell growth and differentiation. Unfortunately, direct addition of ES cells to stirred suspension culture results in significant cell agglomeration and consequently poor cell growth and differentiation [11].

We primarily used mouse ES cells to investigate the process of EB agglomeration and to develop a method for overcoming this cell culture issue. We then verified the relevance and application of these findings for human ES cells. A two-step mechanism for mouse EB agglomeration was elucidated. First, cell-cell adhesion molecule E-cadherin was determined to mediate attachment between neighboring EBs. Following attachment, cells actively migrated and remodeled, assimilating cells into a single spheroid. This mechanism was found to be common to human EBs, and these findings were consistent with the observation that blastocyst-stage human embryos constitutively express E-cadherin [13]. To control cell-cell interactions, mouse and human ES cells were encapsulated in size-controlled agarose hydrogel capsules. Encapsulation permitted the use of high-cell-density culture and enabled EB formation and differentiation to hematopoietic cells in controllable stirred-suspension bioreactors. The importance of this culture system was demonstrated using oxygen tension as an extrinsically controlled inductive signal for hematopoietic development. Hematopoietic progenitor yield was significantly greater in cultures maintained at 4% versus 20% oxygen tension. Together these results demonstrate our capacity to produce scalable quantities of human hematopoietic progenitor cells from human ES cells in bioreactors.

Materials and Methods

ES Cell Culture and Hematopoietic Cell Assays

Maintenance of mouse R1 [14] and YC5 [15] ES cells, preparation of cells for flow cytometry, and preparation of hematopoietic colony-forming cell assay were previously described in *Dang et al.* (2002) [11]. Maintenance of human ES cells (H9.2 and I6) and EB formation were also previously described [10].

Agglomeration of Two EBs

Mouse R1 and YC5 ES cell spheroids (also referred to as EBs for simplicity) cultured in ES media containing leukemia inhibitory factor (LIF; Chemicon; Temecula, CA) were formed in hanging drop culture initiated with 100 ES cells/drop for 24 hours and transferred into nontreated conical 96-well plates (Sarstedt; Newton, NC) containing 100 μl of mouse ES cell medium. One YC5 EB and one R1 EB of the same size were placed together in each well. The plates were gently agitated until the two EBs came in contact with one another. Starting at time 0, photos were taken every 4 hours to record EB interactions.

Test wells were filled with 100 μl of mouse ES cell medium with 40 μg/ml à-mouse E-cadherin (U3254; Sigma; St. Louis, MO), 50 μg/ml Cytochalasin D (Sigma), or 50 μg/ml mitomycin C (Sigma). EBs were incubated separately for 2 hours in their respective solutions before transferring YC5 EBs into the R1 EB wells.

Human EB agglomeration was similarly studied. We placed two human EBs within the same well containing 100 μl of human ES media and observed agglomeration of the EBs over time. Human EBs treated with 40 μg/ml α-human E-cadherin (67A4; Chemicon) were incubated separately for 2 hours before transfer into agglomeration cultures.

Encapsulation Process

Mouse ES cell aggregates were formed by generating a single cell suspension of 3×10^5 cells/ml in ES cell media and allowing cells to aggregate for 1 day. Human ES cell aggregates were formed by partial dissociation by incubating human ES cell maintenance cultures for 20 minutes with 2 mg/ml collagenase B (Sigma). ES cell aggregates were collected and added to molten 2% (weight) low-gelling-temperature agarose (type VII; Sigma) in phosphate buffered saline (PBS; GIBCO-BRL; Rockville, MD) at 2×10^6 cells/ml. The molten agarose mixture was dispensed into 200-centistroke

viscosity dimethylpolysiloxane (DMPS; Sigma) at 37°C and subjected to impeller shearing using the CellSys Microdrop Maker (One Cell Systems; Cambridge, MA) to create agarose hydrogel capsules [16]. Microcapsules were washed twice with Hank's buffered saline solution (HBSS; GIBCO-BRL) and suspended in the appropriate ES cell differentiation media.

Bioreactor Culture

We used the Cellferm-pro system (DasGip; Julich, Germany) for stirred-suspension culture of encapsulated ES cells under controlled conditions. Cellferm-pro consisted of a control, monitoring, dosing, gassing, and cultivation system. We simultaneously operated four pH- and dissolved-oxygen-regulated 400-ml vessels in batch culture mode. Vessels were filled with 200 ml of ES cell media without LIF and inoculated with 5×10^5 ES cells (12,500 ES-cell-containing capsules), achieving a starting cell density of 2.5×10^3 ES cells/ml (60 ES-cell-containing capsules/ml). Cells were cultured for 7 days before harvesting and analyzed by cell counting, flow cytometry, and myeloid-erythroid colony-forming cell assay.

Results

E-Cadherin-Mediated Cell Aggregation

We studied the process of EB agglomeration by quantitatively tracking the fusion of two EBs or two ES cell spheroids (also referred to as EBs for simplicity). To allow observation of cell mixing, one EB generated from wild type R1 mouse ES cells was agglomerated with one EB generated from YC5-R1 [15] mouse ES cells (that constitutively express the yellow fluorescent protein). We calculated the degree of agglomeration (DOA) as the ratio of the interface diameter to the overall length of the two-EB system (Fig. 1A). We measured DOA at regular time intervals and determined initial EB size (50, 100, 400 ES cells/EB) to have no measurable effect on the kinetics of this process (data not shown). Complete agglomeration (DOA >90%) was achieved after approximately 16 hours and complete cell mixing (homogenous fluorescence intensity) after 48 hours (Fig. 1B).

We investigated the mechanism of mouse ES cell aggregation and EB agglomeration using various loss-of-function treatments (Fig. 1C). Blocking E-cadherin cell-adhesion molecules on the EB surface with α-mouse E-cadherin antibodies significantly inhibited EB agglomeration ($p = 0.021$ at 24 hours). This result supports the observation that homozygous E-cadherin-null ES cells are unable to aggregate [17]. Treatment of EBs with cytochalasin D also impaired agglomeration ($p = 0.002$ at 24 hours). Cytochalasin D inhibits actin-dependent processes including cell migration, which suggests that EB agglomeration is an active process and not a result of passive cell diffusion [18]. Based on these results, we proposed a two-step mechanism for EB agglomeration: first, neighboring EBs collide and homophilic E-cadherin molecules adhere EBs together. Cells then actively migrate and remodel the structure until all cells are assimilated into a single spheroid. Impairing either E-cadherin-mediated EB attachment or cell migration inhibits EB agglomeration (Fig. 1C).

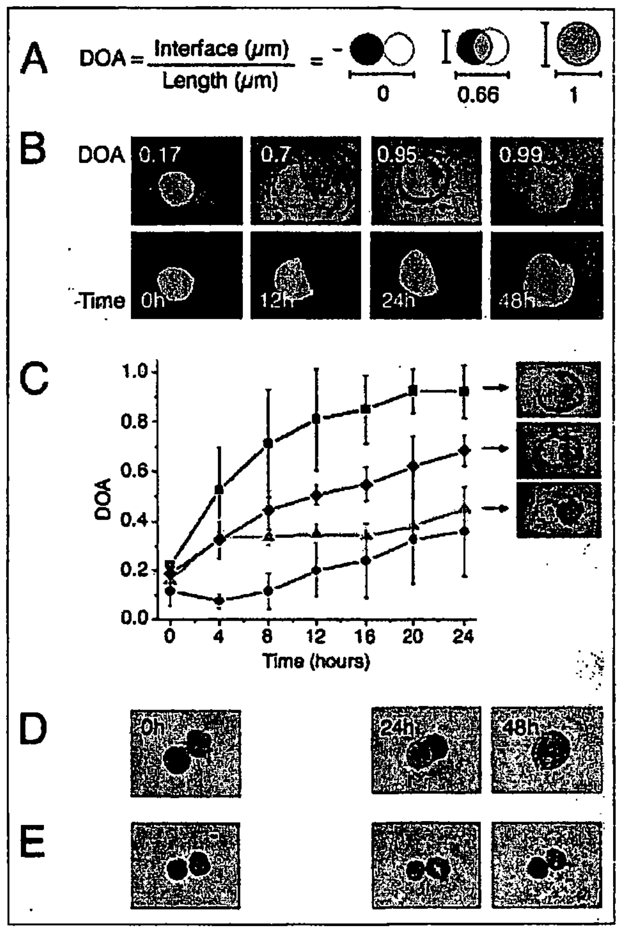
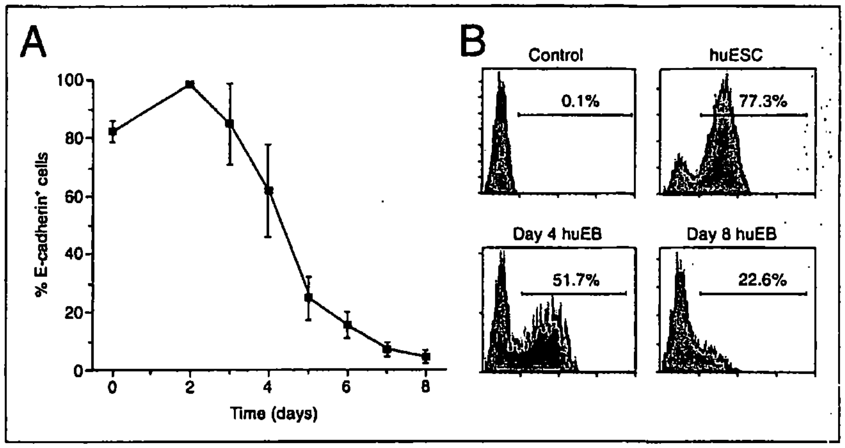


Figure 1. EB agglomeration is initiated by E-cadherin-mediated cell attachment. A) DOA was calculated by dividing the interface diameter by the overall length of the two-EB system. B) Untreated mouse EBs agglomerating over time with calculated DOA at each time interval. Photos taken at 200× magnification. C) Linear graph of DOA (y-axis) versus time (x-axis), n = 5. Compared with the control (-■-), mouse EB agglomeration is impaired by treatment of EBs with α-mouse E-cadherin-blocking antibody (-◆-) and with cytochalasin D (-▲-). Day 6 EBs with downregulated E-cadherin expression (-●-) did not agglomerate. D) Untreated human EBs agglomerating over time. Photos taken at 100× magnification. E) Human EB agglomeration is inhibited by treatment with α-human E-cadherin-blocking antibody. Photos taken at 100× magnification.

Figure 2. A) Percent E-cadherin-expressing cells (y-axis) versus time (x-axis). Mouse EBs differentiated for 3 or fewer days express E-cadherin (>80% cells), correlating with EB agglomeration, n = 3. B) Flow cytometric histogram plots showing percentage of differentiating human ES cells (huESC and HuEB) expressing E-cadherin over time.



To determine whether this mechanism of EB agglomeration was relevant to the human EB system, we performed similar agglomeration studies with human EBs. Untreated human EBs agglomerated more slowly than mouse EBs, requiring 48 hours for complete agglomeration (Fig. 1D). Similar to mouse EBs, agglomeration of human EBs was inhibited by treatment with α-human E-cadherin antibodies (Fig. 1E).

E-cadherin expression by ES cells is downregulated as the cells differentiate [19]. Using flow cytometry, E-cadherin expression was tracked in differentiating EBs over time. Expression of E-cadherin by mouse ES cells remained high (>80%) over the first 3 days of differentiation before being downregulated to approximately 25% by day 5 of differentiation (Fig. 2A). Human ES cells (huESC) also expressed E-cadherin that was downregulated as cells differentiated over time (Fig. 2B). As expected, E-cadherin expression correlated with the rate of EB agglomeration. Mouse EBs differentiated for three or fewer days agglomerated at the same rate as the control whereas day 6 mouse EBs did not agglomerate (Fig. 1C). These results were consistent with the observation that mouse ES cells placed directly into stirred culture aggregated into large cell clumps, whereas mouse EBs grown in static culture for a minimum of 4 days could be transferred to stirred culture with little agglomeration [11].

Control of Cell Aggregation by Encapsulation

Mouse ES cell aggregates (20 to 50 ES cells per aggregate) were individually encapsulated in agarose hydrogel capsules that permitted EB formation while physically preventing EB agglomeration. Capsule diameters between 100-150 μm were designed to encapsulate cells for the first 4 days of differentiation culture when E-cadherin expression remained high (Fig. 3A). EBs emerged from the capsules at an efficiency of 85% ± 10%. The encapsulation procedure was also readily adapted for human ES cell application. To ensure efficient human EB formation, larger ES cell aggregates (1,000-5,000 ES cells per aggregate) were required. These were encapsulated in 200-300 μm diameter capsules designed to retain EBs for the first 8 days of differentiation culture (Fig. 3B). Human EBs emerged from these capsules at an efficiency of 42% ± 15%.

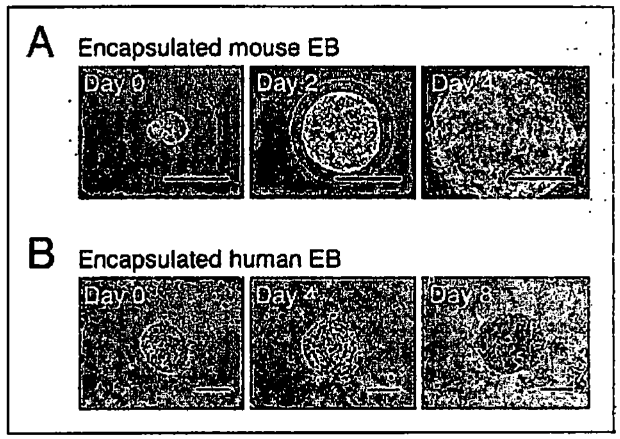


Figure 3. ES cell aggregates were encapsulated in agarose hydrogel microcapsules designed to contain developing EBs while E-cadherin expression remained high. A) Mouse EBs emerge from capsules after 4 days. B) Human EBs emerge from capsules after 8 days. Scale bars = 100 μm.

We first tested the efficacy of the encapsulation approach for controlling EB agglomeration in high-cell-density (input 10^5 mouse ES cells/ml) static cultures. We compared changes in the number of cell aggregates over time between encapsulated and nonencapsulated cells. Non-encapsulated mouse ES cells began culture as individual cells that agglomerated, reducing the number of cell aggregates over time and resulting in varying aggregate sizes (Fig. 4A). In contrast, encapsulation maintained a consistent number of cell aggregates of more uniform size by preventing mouse EB agglomeration (Fig. 4B). After 4 days, encapsulated cultures contained 10 times the number of cell aggregates as non-encapsulated cultures. Having downregulated E-cadherin at

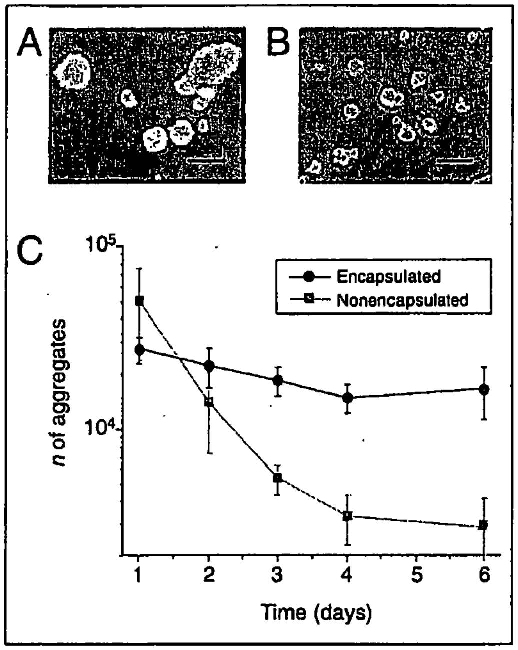


Figure 4. ***Encapsulation prevents EB agglomeration.*** *A) Comparison of total cell aggregate number over time between encapsulated and nonencapsulated mouse ES cells in static culture initiated with 10^5 ES cells/ml,* n *= 3. B) Sample photographs of nonencapsulated and C) encapsulated mouse cell aggregates after 2 days of culture. Scale bars = 100 µm.*

this point, mouse EBs emerged from their capsules and did not aggregate (Fig. 4C). We had previously reported that cell agglomeration, beyond that required for mouse EB formation, negatively affects cell yield [11]. Consistent with this observation, cell expansion after four days of encapsulated culture was significantly higher (23 ± 3-fold over input) than non-encapsulated culture (12 ± 3-fold over input), $p = 0.007$.

We next evaluated cell growth and differentiation potential of encapsulated EBs in stirred culture. EB formation efficiency and cell growth of encapsulated mouse ES cells in stirred culture were similar to standard (non-encapsulated, static) liquid suspension cultures initiated at a typical cell density of 10^4 ES cells/ml (Table 1). This indicated that encapsulation and stirring-related shear stress (at 50 revolutions per minute [rpm] using a glass ball stirrer) did not affect cell yield. Importantly, the differentiation capacity of EBs was also unaffected. The focus of this report involves the generation of hematopoietic progenitors, although other cell types such as cardiac and neural cells were similarly unaffected by encapsulated stirred culture (data not shown). Hematopoietic progenitor frequency was measured by flow cytometric analysis of CD34 and CD45 expression (Fig. 5) and by myeloid-erythroid colony-forming cell (CFC) assays. These phenotypic and functional assays demonstrated that neither the encapsulation process nor encapsulation combined with stirred culture significantly affected hematopoietic progenitor generation when compared with standard liquid suspension culture (Table 2).

Table 1. Cell fold expansion in different culture systems

	Static control	Encapsulated stirred	Non-encapsulated stirred
Fold expansion	61 ± 11	61 ± 10	< 1

Cultures were initiated with 10^4 R1 ES cells/ml, and cell fold expansion was measured after 7 days, $n = 5$.

Controlled ES Cell Differentiation Culture

Process control is most easily employed in stirred-suspension culture because point measurements accurately reflect bulk media conditions, and changes in culture conditions can be rapidly implemented. We used these capabilities to maintain differentiating mouse ES cells at different oxygen

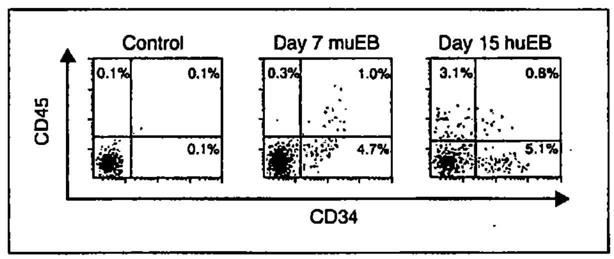


Figure 5. ***Representative flow cytometric analysis plots of cell surface marker CD34 (x-axis) and CD45 (y-axis) expression after 7 days of mouse ES cell (MuEB) differentiation culture and 15 days of human ES cell (HuEB) differentiation culture.***

Table 2. Comparison of mouse hematopoietic progenitor frequency in various encapsulated (E) culture systems with standard liquid suspension culture (LSC). Flow cytometric analysis of CD34 and CD45 coexpression and myeloid-erythroid colony-forming cell (CFC) assay results are tabulated. Hypoxic and normoxic populations were compared by Student's t test, and the calculated p value is shown, $n = 5$.

Culture system	$CD34^+CD45^+$	*p* value	CFCs (per 10^5 cells)	*p* value
LSC static normoxic	1.1% ± 0.4%		14 ± 6	
E static normoxic	1.0% ± 0.5%	0.85	10 ± 3	0.22
E stirred normoxic	0.8% ± 0.2%	0.13	8 ± 2	0.09
E stirred hypoxic	3.6% ± 2%	0.008	54 ± 21	0.0006

Neither the encapsulation process nor stirring significantly affected hematopoietic progenitor development (significance level $p = 0.05$). However, the ability to culture cells in controlled hypoxic conditions greatly improved hematopoietic progenitor frequency.

tensions. Dissolved oxygen and pH were measured online and maintained at normoxic 20% or hypoxic 4% oxygen tension (Fig. 6A) and pH 7.4.

Hematopoietic progenitor generation was compared between normoxic and hypoxic cultures after 7 days of differentiation. Hematopoietic progenitor frequency, measured by flow cytometric analysis of CD34 and CD45 expression and by myeloid-erythroid CFC assay, was significantly higher in hypoxic versus normoxic culture (Table 2). In terms of numerical CFC yield, 17,000 ± 4,700 CFCs were generated from an input of 5×10^5 ES cells per hypoxic bioreactor compared with 2,000 ± 600 CFCs per normoxic bioreactor (Fig. 6B). Increase in CFC frequency and yield was due to approximately equal proportional increases in lineage-restricted hematopoietic progenitors, although numerically, erythroid progenitors were responsible for most of the CFC increase under hypoxic conditions.

Discussion

The ability to generate large numbers of ES-derived cells in controlled in vitro conditions is an important step toward their clinical application. Stirred-suspension bioreactors are ideal for this purpose because they can be used to generate scalable quantities of cells, facilitate process control strategies, and simplify the cell production process.

Controlling cell aggregation was necessary for EB formation and growth in stirred-suspension bioreactors. Strategies to prevent global cell aggregation, including addition of dextran sulfate, polyvinyl sulfate, and the use of high impeller rpm [20, 21] were unsuccessful because they prevented initial ES cell aggregation required to induce EB formation and/or disrupted EB architecture, thus impacting cell proliferation, viability, and differentiation (data not shown). Our investigation showed that blocking the function of E-cadherin could impair EB agglomeration. E-cadherin has been implicated in tissue organization and regulation of gene expression—both of which affect cell differentiation [17]. Use of blocking antibodies in culture would not only be economically impractical, but also may adversely affect cell differentiation.

We demonstrated that mass encapsulation of ES cells under defined conditions (i.e., cells per EB and capsule sizes) was a practical method for controlling cell aggregation in vitro. Our system has advantages over other EB formation systems [11, 22] because it allows for EB development in controlled stirred-suspension bioreactors. Mouse ES cells were used to study the process of EB agglomeration and to optimize our encapsulation system, which we then validated for the human ES cell system. Contrary to differences in the reported conditions for the

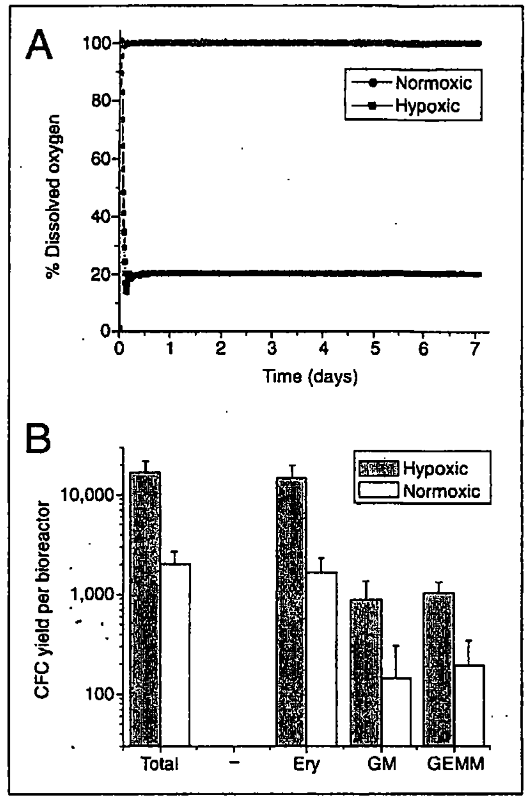


Figure 6. Generation of mouse hematopoietic progenitors in hypoxic and normoxic stirred-suspension cultures. A) Sample dissolved oxygen profiles in controlled stirred-suspension bioreactors. B) Yield of colony forming cells (CFCs) per bioreactor (initated with 5×10^5 ES cells). Total CFC yield per bioreactor as well as lineage-specific yield of CFC erythrocyte (Ery), granulocyte-macrophage (GM), and granulocyte-erythrocyte-megakaryocyte-macrophage (GEMM) are shown, n = 5.

maintenance of undifferentiated ES cells, our results suggest that similar mechanisms may govern ES cell differentiation in mouse and human systems.

We determined that encapsulation of ES cells was necessary for efficient EB formation, cell proliferation, and differentiation in stirred-suspension bioreactors. This enabled, for the first time, the investigation of the controlled manipulation of exogenous factor influences on the differentiation of ES cells. Low-oxygen conditions have been reported to induce expression of various genes in differentiating ES cells, including vascular endothelial growth factor (VEGF) [23, 24] and glycolytic enzymes such as aldalose A [23] via a hypoxia inducible factor-1 (HIF-1)-mediated response [24]. Increased hematopoietic progenitor cell frequency was also reported [24]. We used online oxygen tension measurement and automated gas mix control to maintain cultures at either 4% or 20% oxygen tension. Unlike in

previous investigations, we were able to isolate the effects of oxygen tension (independent of other medium parameters including pH) by controlling pH at a set point value of 7.4, thereby preventing glycolytic acidification of the media. We confirmed that low-oxygen conditions significantly improved hematopoietic progenitor cell frequency and verified that numerical yield of these cells was also improved. The ability to accurately measure and control culture conditions in stirred-suspension bioreactors will be a valuable tool for understanding and optimizing delivery profiles of exogenous factors that affect ES cell differentiation. For example, we have determined that providing hypoxic conditions between days 3 and 7 of mouse ES cell differentiation is critical for the increase in hematopoietic progenitor yield (data not shown). Additionally, the ability to culture encapsulated ES cells at high density may facilitate identification and quantification of secreted factors (in addition to VEGF) involved in hypoxic expansion of hematopoietic progenitors.

We demonstrated the utility of our cell production approach by generating clinically relevant numbers of hematopoietic progenitors that may, at the very least, be useful for rapid short-term engraftment of host animals [25]. This production process is readily compatible with the cell selection strategy described by *Klug et al.* (1996) [12] that confers cell-lineage-specific antibiotic resistance. We are currently developing an integrated production and purification process to generate pure populations of cardiac cells using ES cells transfected with a selection plasmid pgk-hygro-myosin heavy chain-neo [26].

Together, these examples and the results presented herein highlight the importance of stirred-suspension culture for optimizing and controlling physicochemical factors that influence cell growth and differentiation. Our results also demonstrate production of scalable quantities of therapeutically useful cell types. In addition, cell encapsulation may prove useful by providing a scaffold onto which cytokines or extracellular matrix proteins can be attached. Delivery of bioactive molecules in this highly localized manner may allow for the provision of developmentally relevant gradients of molecules in scalable culture and will certainly provide a cost-effective alternative to maintaining the entire bulk media at a specific concentration. Ultimately, encapsulated stirred culture may provide the leverage to control most exogenous factors that affect ES cell growth and differentiation including cell-cell interactions, physicochemical factors, and cytokine delivery.

Acknowledgments

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Exhibit 3

Development of a Perfusion Fed Bioreactor for Embryonic Stem Cell-Derived Cardiomyocyte Generation: Oxygen-Mediated Enhancement of Cardiomyocyte Output

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Abstract: Cell transplantation is emerging as a promising new approach to replace scarred, nonfunctional myocardium in a diseased heart. At present, however, generating the numbers of donor cardiomyocytes required to develop and test animal models is a major limitation. Embryonic stem (ES) cells may be a promising source for therapeutic applications, potentially providing sufficient numbers of functionally relevant cells for transplantation into a variety of organs. We developed a single-step bioprocess for ES cell-derived cardiomyocyte production that enables both medium perfusion and direct monitoring and control of dissolved oxygen. Implementation of the bioprocess required combining methods to prevent ES cell aggregation (hydrogel encapsulation) and to purify for cardiomyocytes from the heterogeneous cell populations (genetic selection), with medium perfusion in a controlled bioreactor environment. We used this bioprocess to investigate the effects of oxygen on cardiomyocyte generation. Parallel vessels (250 mL culture volume) were run under normoxic (20% oxygen tension) or hypoxic (4% oxygen tension) conditions. After 14 days of differentiation (including 5 days of selection), the cardiomyocyte yield per input ES cell achieved in hypoxic vessels was 3.77 ± 0.13, higher than has previously been reported. We have developed a bioprocess that improves the efficiency of ES cell-derived cardiomyocyte production, and allows the investigation of bioprocess parameters on ES cell-derived cardiomyogenesis. Using this system we have demonstrated that medium oxygen tension is a culture parameter that can be manipulated to improve cardiomyocyte yield. © 2005 Wiley Periodicals, Inc.

Keywords: bioreactor design; stem cells; cardiomyocytes; oxygen; tissue engineering

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INTRODUCTION

Adult cardiomyocytes are terminally differentiated cells with limited capacity for cell division. Currently, the only effective treatment for patients suffering from severe heart failure is organ transplantation. The successful engraftment of several cell types into the heart (Orlic et al., 2001a; Taylor et al., 1998) has given rise to the concept of cell transplantation as a treatment for heart failure. Although a number of contractile cell types have appeared to improve heart function upon transplantation (Orlic et al., 2001a,b; Taylor et al., 1998), cardiomyocytes are the ideal donor cell due to their inherent electrical and physiological properties. However, obtaining adult cardiomyocytes in sufficient quantities for effective transplantation has been hindered by the limited availability and proliferative capacity of these cells (Soonpaa et al., 1996). Embryonic stem (ES) cells, pluripotent cells isolated from the inner cell mass of the developing mammalian blastocyst, self-renew indefinitely while retaining their capacity to differentiate into cell lineages of all three primary germ layers (Evans and Kaufman, 1981), including cardiomyocytes (Maltsev et al., 1993). ES cell culture may be a promising tool for cell therapy, potentially providing a renewable cell source for transplantation into a variety of organs.

In vitro mouse ES cell differentiation typically requires an initial aggregation step to form spherical cell clusters called embryoid bodies (EBs). It has been well established, by our group and others, that cardiomyocytes derived from EB differentiation display properties characteristic of functional (fetal) cardiac cells. Differentiating EBs recapitulate many aspects of cardiogenesis in the embryo, displaying protein expression profiles that parallel the developmental expression pattern exhibited during in vivo

heart formation starting with cardiac transcription factors, followed by chamber-specific proteins, sarcomeric proteins, and ion exchanger proteins (Czyz and Wobus, 2001; Sachinidis et al., 2003). These cells display electrophysiological responses, expressing pacemaker-, atrial-, and ventricular-like action potentials in a developmentally regulated manner (Hescheler et al., 1999; Maltsev et al., 1993).

Herein we report the development of a scalable bioprocess for the culture of ES cell-derived cardiomyocytes in a medium-perfused bioreactor system (Fig. 1). Accomplishing this endpoint required incorporating methods to address specific challenges associated with high-density stirred suspension ES cell differentiation systems. Recognizing that ES cells express surface molecules that promote aggregation between EBs (EB agglomeration), thus inhibiting cell growth and/or differentiation (Dang et al., 2002), we used a hydrogel encapsulation approach (Dang et al., 2004; Magyar et al., 2001) to control EB development during stirred suspension culture. Encapsulation not only permitted direct differentiation of ES cells in stirred suspension, it also significantly improved cell production. Cardiomyocytes typically constitute less than 5% (Klug et al., 1996) of all cells during EB-based differentiation. Furthermore, EB differentiation is often incomplete, resulting in the persistence of undifferentiated ES cells (Erdo et al., 2003; Gulbins et al., 2002; Hilberg and Wagner, 1992). To purify for cardiomyocytes, and to deplete undifferentiated cells, we and others have used a genetic selection technique (Klug et al., 1996; Li et al., 1998; Marchetti et al., 2002; Zandstra et al., 2003) whereby a transgene, encoding neomycin resistance driven by a myosin heavy chain (MHC) promoter, is stably transfected into ES cells. This technique has been demonstrated to efficiently enrich ES cell-derived cardiomyocytes to greater than 70% (Zandstra et al., 2003). This report additionally begins to address the low cardiomyocyte yield typically achieved in spontaneously differentiating ES cell cultures, demonstrating cardiomyogenesis is enhanced during hypoxic (4% oxygen tension) culture. Taken together, this first demonstration of a one-step controllable perfusion bioreactor system for the generation of embryonic stem cell derivatives should serve as a foundation for ES cell bioprocess development.

MATERIALS AND METHODS

ES Cell Maintenance

The D3 (Doetschman et al., 1985) mouse ES cell line was used in this study, with encapsulation and cardiomyocyte derivation results consistent with the R1 and CM7/1 (Zandstra et al., 2003) (a clone of the J1 ES cell line) ES cell lines, respectively. ES cell maintenance has been previously described (Zandstra et al., 2003). Undifferentiated cells were bulk transfected, via electroporation, with a plasmid carrying both an α-myosin heavy chain promoter in front of the neomycin phosphotransferase gene (MHC-neo^r) and a phosphoglycerate kinase in front of a hygromycin-

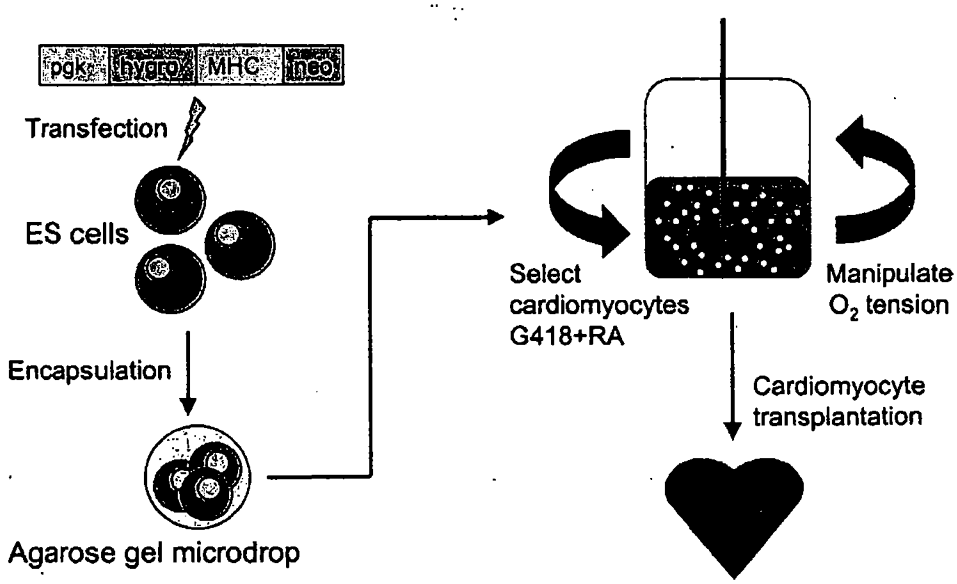


Figure 1. Overview of bioprocess for ES cell-derived cardiomyocytes. Transfected ES cells are encapsulated in hydrogel microcapsules and directly inoculated into spinner cultures. Differentiation proceeds until d9 (oxygen tension can be optimized for improved cardiomyocyte yield). Cultures are treated with G418 and RA to select for cardiomyocytes. On d14, an enriched cardiomyocyte population is harvested that can be used in downstream applications such as tissue engineering and model studies.

resistant (PGK-hygro^r) gene in a common pUCBM20 vector backbone (Boehringer-Mannheim, Germany) (Zandstra et al., 2003).

ES Cell Differentiation Cultures

Unencapsulated Suspension Cultures

Stirred suspension differentiation of ES cells to cardiomyocytes was performed as previously described (Zandstra et al., 2003). This system consists of forming EBs under static conditions, to prevent EB agglomeration, and then transferring the EBs to stirred suspension on day 4 (d4) of differentiation. This system will be referred to as the static/spinner flask (S/SF) system.

Encapsulated Suspension Cultures

Mouse ES cell aggregates were formed by generating a single-cell suspension of 3×10^5 cells/mL in ES cell media and allowing cells to aggregate for 1 day. ES cell aggregates were dispersed in molten 1.5% (weight) low-gelling-temperature agarose (type VII, Sigma, St. Louis, MO) in Dulbecco's phosphate-buffered saline (PBS, GIBCO-BRL, Grand Island, NY) at 2×10^6 cells/mL. The molten agarose mixture was dispensed into 200-centistoke viscosity dimethylpolysiloxane (DMPS, Sigma) at 37°C and subjected to impeller shearing using the CellSys Microdrop Maker (One Cell Systems) to create agarose hydrogel microcapsules (Ryan et al., 1995). Microcapsules were washed twice with Hank's buffered saline solution (HBSS, GIBCO-BRL) and suspended in the appropriate ES cell differentiation media. The microcapsules were inoculated into 250-mL spinner flasks (Bellco Glass, Vineland, NJ) at a density of 4,000 cell/mL, as it was observed that this density resulted in exponential cell growth to our d9 target density (1×10^6 cell/mL), at which point cardiomyocyte selection was initiated by adding Geneticin (G418, GIBCO-BRL) (400 μg/mL) to kill noncardiomyocytes and retinoic acid (RA, Sigma) (10^{-9}M) which has been shown to deplete undifferentiated ES cells and improve cardiomyocyte yield (Zandstra et al., 2003). The encapsulated ES cell aggregates form EBs that differentiate and grow within the capsule until ~d4, at which time most of the EBs have grown large enough to emerge from the capsules (Dang et al., 2004).

Bioreactor Culture

We used DasGip's cellferm-pro culture system (www.dasgip.com), a parallel cultivation system that is capable of monitoring and controlling oxygen tension and pH in four parallel 500-mL vessels. Using an 8-head multipump (inlet and outlet flow to the four vessels), continuous medium perfusion was implemented by attaching a settling tube to the feed outlet. The diameter of the settling tube (>1.38 mm) was specified so that the velocity of the medium outlet was slower than 150 cm/hr, the settling velocity of the EBs on d9. Iscove's Modified Dulbecco's Medium (IMDM, University of Toronto Media Prep) differentiation medium was fed to the cultures at a rate of one-quarter reactor volume replacement per day.

The bioreactors were maintained under either normoxic (20% O_2 tension) or hypoxic (4% O_2 tension) conditions to d9. On d9, selection was initiated by supplementing the feed with 400 μg/mL G418 and 10^{-9}M RA and the feed rate was increased to three-quarter reactor volume replacement per day to remove the dead cell debris produced by selection, and to maintain G418 and RA concentration. During selection all vessels were controlled at 20% oxygen tension.

C2C12 Cell Culture

Two different culture media were used to induce C2C12 differentiation into myotubes. C2C12 culture medium (nondifferentiating) was used for seeding and consisted of 90% Dulbecco's Modified Eagle Medium (DMEM, GIBCO-BRL), 10% FBS, 4 mM L-glutamine (Invitrogen, La Jolla, CA), 2 mM L-penicillin (50 IU/mL, Invitrogen), and streptomycin (50 μg/mL, Invitrogen). C2C12 differentiation medium consisted of 98% DMEM, 2% horse serum (HS, GIBCO-BRL), 4 mM L-glutamine, penicillin (50 IU/mL), and streptomycin (50 μg/mL). C2C12 cells were seeded in a 24-well plate at a density of 50,000 cells per well in C2C12 culture media. The following day the cells were washed once with PBS and then cultured in C2C12 differentiation media. Cells were maintained in differentiation media for the next 4 days, replacing the medium every other day.

Immunofluorescence of Whole-Mount Embryoid Bodies and C2C12 Cells

Intracellular antibodies used for immunostaining whole EBs and C2C12 cells required the fixation and permeabilization of the cells within the EBs. EBs were fixed using IntraPrep Fixation Reagent 1 (Immunotech, Westbrook, ME) overnight, then washed by resuspension in 1.5 mL of HBSS containing 2% fetal bovine serum (FBS, Hyclone, Logan, UT) (HF) every hour for a total of five washes. Fixed EBs were permeabilized in IntraPrep Reagent 2 for 1 h and incubated with either anti-sarcomeric myosin heavy chain (MF20, 1:10, DSHB), anti-α-actinin (EA-53, 1:800, Sigma) or anti-nebulin (NB-2, 1:400, Sigma) antibodies overnight. Stained EBs were washed five times in HF and then incubated with secondary antibody and nuclear dye (1:100, FITC-conjugated for MF20 stained EBs, PE-conjugated for NB-2 and EA-53 stained EBs, 1:100, 7-AAD for nuclear dye) overnight and washed the next day. An Olympus inverted IX70 microscope was used in combination with the FV-300 confocal microscope-

scanning unit to observe EBs. The excitation lasers used were 488 nm argon and 543 nm HeNe.

C2C12 cells were washed once in PBS and fixed in 3.7% formaldehyde (EMD Chemicals, San Diego, CA) in PBS for 15 min at 37°C. The cells were subsequently washed three times with PBS and permeabilized in 100% methanol (EMD Chemicals) for 2 min at room temperature. The cells were then washed three times with PBS and blocked overnight with 10% FBS in PBS at 4°C. The next day C2C12 cells were stained with NB-2 for 2 h at room temperature followed by three washes with PBS. The cells were then incubated with Alexaflor 546 goat antimouse TRITC (1:200, Molecular Probes, Eugene, OR) and Hoescht 33342 (1:100, Sigma) at room temperature. The cells were imaged using Arrayscan II high through-put fluorescent microscope (Cellomics).

RT-PCR

Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma). RNA was quantified by a UV spectrophotometer and 0.5 μg RNA was used in each RT-PCR reaction (One-Step RT-PCR kit, Qiagen, Chatsworth, CA). The oligonucleotide primers for MHC and MLC-2v amplification were respectively 5′-CTGATGGCACAGAAGATGCT-3′ and 5′-GTTCAGGATGCGATACCTCT-3′, and 5′-GAACTCTCCAGAGGTGGCAA-3′ and 5′-CCTCTCTGCTTGTGTGGTCA-3′. The PCR amplification conditions were 30 cycles of 30 sec at 94°C, 30 sec at 52°C. The sizes of the anticipated RT-PCR amplification products were 1,058 and 422 bp for MHC and MLC-2v, respectively. Equivalent loading was verified by amplification of α-actin, and primer specificity was verified by RT-free amplification in the second reaction (not shown).

Glucose and Lactate Analysis

Medium samples were analyzed using the BioProfile 200 blood gas analyzer (Nova Biomedical, Waltham, MA). For the semicontinuously fed spinner flasks, samples were collected prior to medium exchange, and then again following medium exchange. For the perfusion fed cultures, medium samples were taken from the feed outlet throughout the course of differentiation.

Flow Cytometry

EBs were dissociated to single cell suspensions as previously described (Zandstra et al., 2003). Cells were double-stained with MF20 and ethidium monoazide (EMA, Molecular Probes), a viability dye that irreversibly binds DNA. Dispersed cells (5×10^5 to 1×10^6) were suspended in 100 μl of HF and 1 μL EMA (0.5 mg/mL EMA) and incubated for 20–30 min exposed to visible light. Samples were washed in HF and the MF20 staining protocol proceeded as previously described (Zandstra et al., 2003).

Statistics

All statistical analyses were performed with Origin 6.1 (OriginLab, Northampton, MA) graphing and data analysis software. All results, generated from at least three independent experiments, were analyzed using a significance level of $P = 0.05$.

RESULTS

Anti-Sarcomeric Myosin Is a Cardiac-Specific Antibody Over the First 14 Days of Differentiation

Sarcomeric myosin heavy chain is a protein expressed in cardiomyocytes as well as skeletal myocytes. To ensure that MF20, an antibody against sarcomeric myosin, could be employed as a marker to screen for cardiomyocytes during the stages of differentiation investigated herein, immunofluorescence of whole-mount EBs was conducted on d9 and d14. EBs were double-stained with either MF20 and EA-53 (skeletal and cardiac myocytes), or MF20 and NB2 (skeletal cells only). Colocalization of MF20 and EA-53 was observed on both d9 and d14, whereas nebulin expression was not observed in either d9 or d14 EBs (Fig. 2A), indicating no differentiation of ES cells into skeletal myocytes during this time frame. Thus, the MF20 antibody is a cardiac-specific marker during the differentiation period in this study.

To further confirm the cardiomyocyte phenotype of these cells we analyzed RNA isolated from cells on d0, d9, and d14. Semiquantitative RT-PCR revealed that the cells cultured in this system exhibit cardiac-specific gene expression (Fig. 2B). Transcripts for MLC-2v, a gene expressed by ventricular cells, were weakly expressed by d9 and strongly expressed by d14, after 5 days of selection. Furthermore, cardiac-specific α-MHC was strongly expressed by d9, when selection was initiated. These characterization studies support other published results (Doevendans et al., 2000; Klug et al., 1995) indicating that sarcomeric myosin heavy chain expression can be used to screen ES cell differentiation to cardiomyocytes in 14-day differentiation cultures.

ES Cell Encapsulation Prevents EB Agglomeration and Does Not Impact Cardiogenic Induction

ES cells express cell adhesion molecules that are downregulated as they differentiate. Due to the high incidence of cell contact in stirred cultures, previous studies (Zandstra et al., 2003) used the S/SF system, a two-step process that incorporates an initial static differentiation period before transferring the EBs to stirred suspension on d4, at which time cell adhesion molecules are sufficiently downregulated. In order to eliminate this static culture step and to differentiate ES cells directly in stirred suspension in a one-step process, we evaluated the capacity for encapsulated EBs (Table I, Fig. 3A) to form cardiomyocytes.

Sarcomeric myosin expression on d9 showed that similar cardiomyocyte frequencies were achieved in encapsulated and S/SF differentiation cultures (Fig. 3B), demonstrating that cardiomyocyte formation proceeds normally in encapsulated EBs.

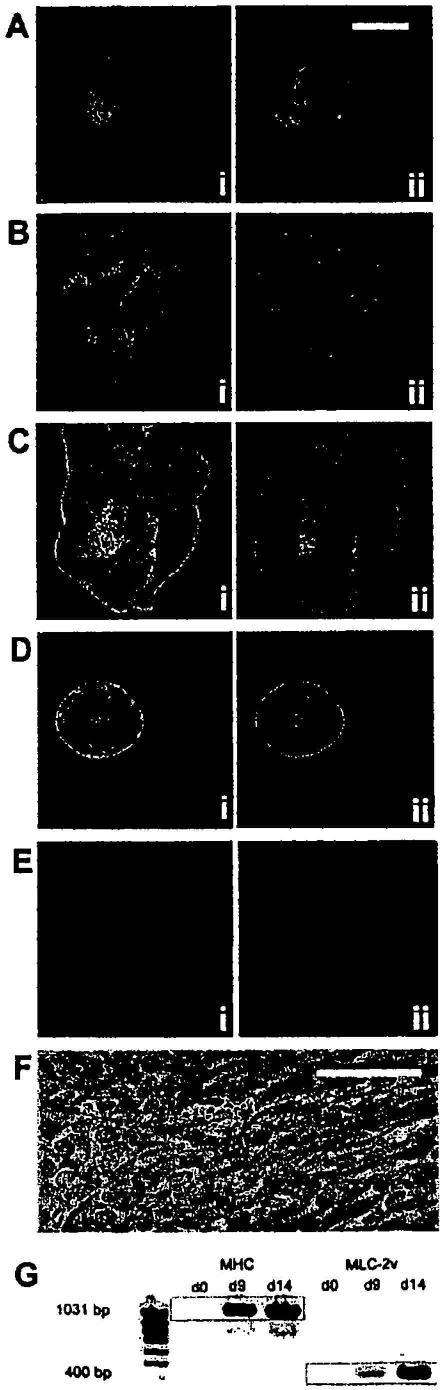


Table I. Cardiomyocyte yield per input ES cell (C/ES) as a function of encapsulation and hypoxic culture.

Study	Condition	C/ES[a]	Fold increase (normalized to control)
Encapsulation	Control (S/SF)[b]	0.16 ± 0.07	
	EC[c]	3.17 ± 0.90	19.8
Oxygen	Control (normoxic)	2.56 ± 0.11	
	Hypoxic	3.77 ± 0.13	1.47

[a]C/ES: cardiomyocyte yield per input ES cell.
[b]S/SF: 2-step static/spinner flask system.
[c]EC: 1-step encapsulation culture system.

Encapsulation Cultures Achieve Higher Cardiomyocyte Yields per Input ES Cell

To reduce undefined interaction effects that may occur at high cell densities, we set a maximum target cell density of 1×10^6 cells/mL, (achieved by d9) in the stirred cultures. In encapsulation cultures, there is an ES cell aggregation step prior to encapsulation and initiating differentiation, whereas in the S/SF system EB formation is initiated with single cells, and thus a higher starting density was required to obtain standard size EBs for cardiomyocyte differentiation. The S/SF cultures required 24×10^6 input ES cells to achieve our target cell density in a 250-mL culture volume. In contrast, only 1×10^6 input ES cells were needed to achieve this target in encapsulated cultures. This represents an improvement in cell production that is over an order of magnitude higher in encapsulated cultures than in the S/SF system (Table I, Fig. 4A). Encapsulation prevented EB agglomeration, suggested by the higher EB numbers (Fig. 4B) despite similar cell number (Fig. 4C), and therefore resulted in more efficient cell growth in differentiating EBs. The greater overall cell expansion in encapsulated cultures improved the cardiomyocyte yield per input ES cell (C/ES) in these studies to 3.17 ± 0.90 vs. 0.16 ± 0.07 in S/SF cultures (Table I, Fig. 4D).

Perfusion Feeding Minimizes Fluctuation of Medium Components

In S/SF cultures, and in typical EB differentiation processes, medium supplementation consists of a half-volume

Figure 2. Immunostaining of EBs revealed that skeletal myocytes were not present in the first 14 days of differentiation. 10× magnification, scale bar = 280 μm. **A:** A d9 EB demonstrating colocalization of (i) MF20 and (ii) EA-53. **B:** A d9 EB demonstrating (i) presence of MF20 staining and (ii) absence of NB-2 staining. **C:** A d14 EB demonstrating colocalization of (i) MF20 and (ii) EA-53. **D:** A d14 EB demonstrating (i) presence of MF20 staining and (ii) absence of NB-2 staining. **E:** Secondary antibody controls for (i) FITC, and (ii) PE. **F:** NB-2 positive control using C2C12 cells. **G:** RT-PCR analysis of MLC-2v, and α-MHC transcription on d0, d9, and d14 of ES cell differentiation in stirred suspension culture (bands highlighted by boxed region).

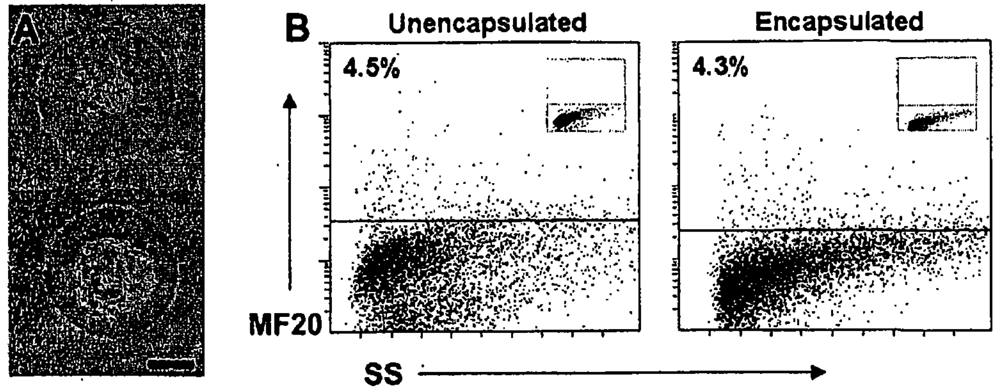


Figure 3. Encapsulated ES cell aggregates form EBs capable of cardiomyocyte differentiation. **A:** Day 0 ES cell aggregates (~ 150 cells/aggregate) encapsulated in agarose hydrogel microcapsules. 10× magnification, scale bar = 70 µm. **B:** Representative MF20 flow cytometry plots of samples from d9 unencapsulated and encapsulated stirred suspension cultures (negative secondary antibody controls, inset).

medium exchange every second day. In the development of our bioprocess we enabled continuous medium perfusion at the same overall feed rate (half-volume every second day) by incorporating a settling tube to retain cellularity. Automated continuous perfusion reduced the significant fluctuations in the concentration of medium components, such as glucose (Fig. 5A) and lactate (Fig. 5B), which resulted from the previous semicontinuous method of medium exchange.

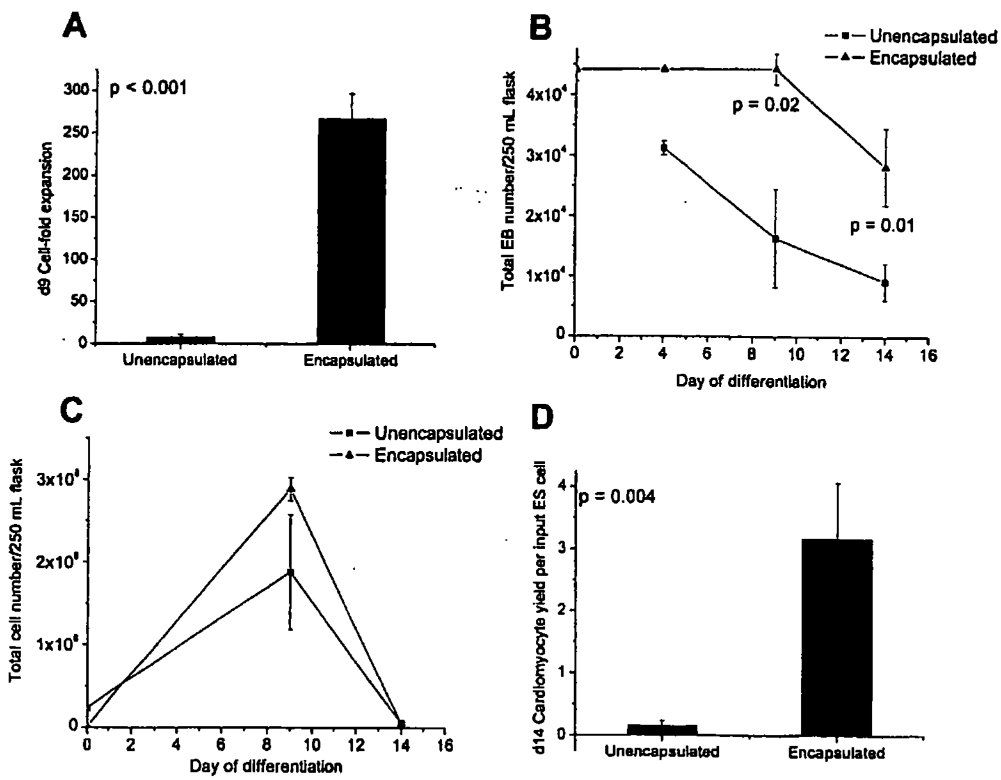


Figure 4. Encapsulating ES cell aggregates resulted in more efficient cell growth leading to higher cardiomyocyte yields per input ES cell. **A:** d9 cell production. **B:** Total EB numbers during the course of differentiation. **C:** Total cell numbers during the course of differentiation. **D:** Cardiomyocyte yield per input ES cell on d14.

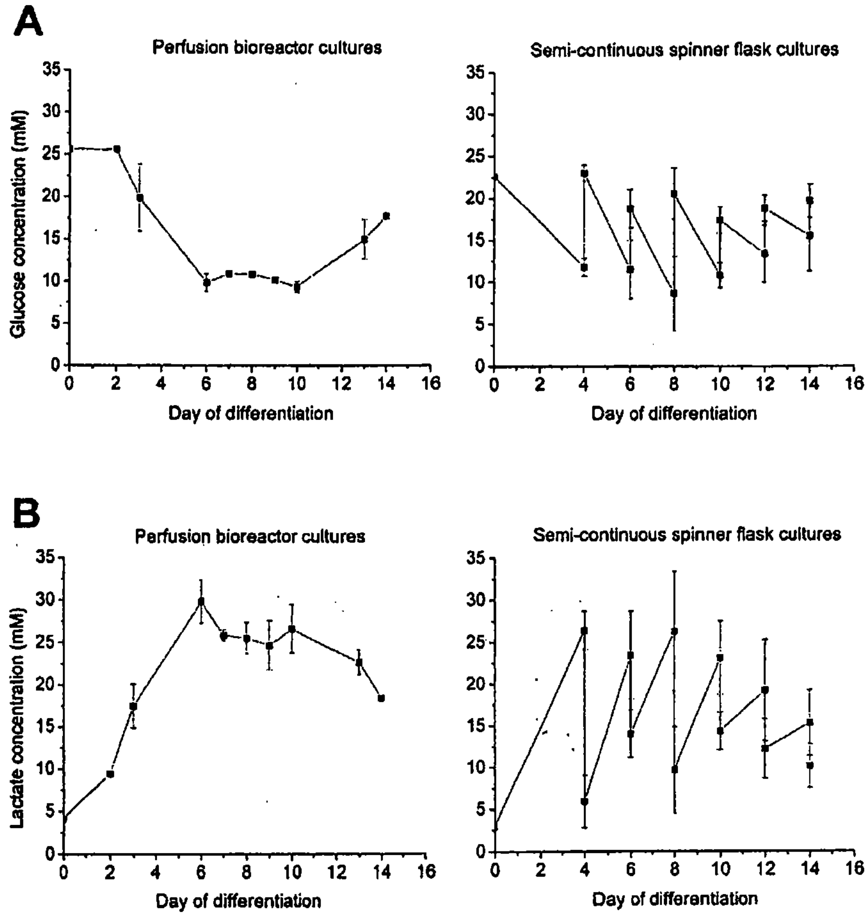
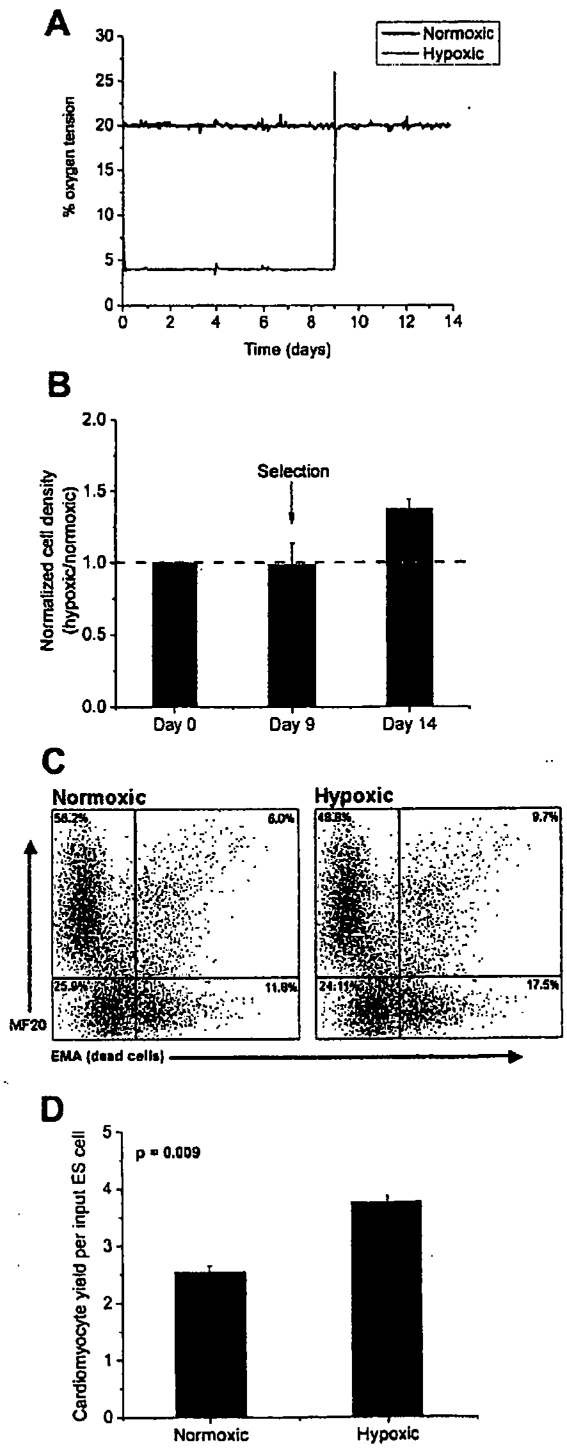


Figure 5. Perfusion feeding reduced fluctuations in the concentrations of medium components. **A:** Glucose concentration over time in perfusion fed and semicontinuous fed culture systems. **B:** Lactate concentration over time in perfusion fed and semicontinuous fed culture systems.

Cardiomyocyte Yield in Differentiating ES Cells Can Be Increased by Manipulating Oxygen Tension

Enabling a single-step bioprocess with automated medium perfusion allowed us to use the DasGip Cellferm-pro bioreactor system's microenvironmental control capacities to investigate the effect of oxygen tension on cardiomyogenesis. Cardiomyocyte yield was compared under normoxic and hypoxic culture conditions. Normoxic cultures were controlled at 20% oxygen tension for the duration of the experiment. Hypoxic conditions were controlled at 4% oxygen based on previous studies suggesting that this level of oxygen is sufficient to activate transcription of hypoxia responsive genes (Adelman et al., 1999; Gassmann et al., 1996). At ~day 7 to day 9 after initiating differentiation (Boheler et al., 2002; Sachinidis et al., 2002) the presence of cardiomyocytes can be observed by the appearance of spontaneous rhythmically contracting areas that increase in number and in area as differentiation proceeds. Therefore, we imposed a hypoxic culture environment, controlled at 4% oxygen tension, until d9 and then maintained oxygen tension at 20% for the remainder of the experiment (Fig. 6A). This design allowed us to focus our investigation on the effects of hypoxia on the initial stages of cardiac development. Both cultures exhibited similar proliferation until d9, indicating that hypoxia does not affect overall cell growth during this stage of differentiation (Fig. 6B). Prior to selection, cardiomyocyte frequency was too low to detect significant differences between the two conditions (data not shown). By d14 (5 days of selection), flow-based MF20 analysis revealed significant enrichment and similar frequencies of cardiomyocytes under both conditions with 66.90 ± 2.12% and 69.28 ± 3.34% cardiomyocytes for normoxic and hypoxic conditions, respectively (Fig. 6C). $3.77 \times 10^6 \pm 0.13 \times 10^6$ cardiomyocytes were present in the hypoxic cultures; more than 1.47 times as many as in normoxic cultures (Table I, Fig. 6D). These results suggest that more cardiomyocytes, or cardiomyocyte precursor cells, were formed by d9 under hypoxic conditions, as similar cardiomyocyte frequencies but higher cell yields were observed than under normoxic conditions (C/ES = 3.77 ± 0.13 vs. 2.56 ± 0.11).



DISCUSSION

Effective cardiomyocyte transplantation requires the successful seeding of sufficient cell numbers in an infarcted or diseased heart. It has been estimated that in large myocardial infarctions, which result in heart failure, 10^8 cardiomyocytes are typically lost (Kehat and Gepstein, 2003). Transplantation studies are important to determine the effects of ES cell-derived cardiomyocyte engraftment on parameters such as survival of donor cells, seeding efficiency, tumor formation, etc. It has been shown that ES cell derived cardiomyocyte grafts survive at the transplantation site for up to 30 days in ectopic transplants (Johkura et al., 2003) and 7 weeks injected directly into the heart (Klug et al., 1996). However, such studies on the transplantation potential of ES cell-derived cardiomyocytes have been limited by the number of donor cells available, with the injection of only 10^4 ES cell-derived cardiomyocytes (Klug et al., 1996) into the site of injury. Herein, we demonstrate that more than 3.5×10^6 cardiomyocytes can be produced in a 250-mL culture volume. This system is readily scalable and preliminary results using this bioprocess in a 1-L bioreactor (Applikon Biobundle) have generated greater than 10^7 cardiomyocytes. Scale-up concerns related to enlarging the system to generate clinically relevant numbers of donor cells may be addressed by running multiple smaller reactors in parallel, or increasing bioreactor size. Because of the significant reduction in input ES cell numbers required using encapsulation, generating sufficient numbers of cells to inoculate a 10-L bioreactor would be relatively simple (~100 million ES cells or 3 to 5 T-75 flasks). We have taken a process that typically occurs in a 2–5 mL Petri dish and enabled a controlled bioreactor-based single-step bioprocess. Current investigations are focused on adapting the system for higher-density cell cultures (>5×10^6 cells per mL, where microenviromental control will be critical to cell survival).

We have previously reported, using the CM7/1 cell line, a clone selectable for increased cardiomyocyte generation, the production of 1.5×10^7 cardiomyocytes from 2.4×10^7 ES cells (C/ES = 0.63) in the S/SF culture system (Zandstra et al., 2003). Another research group, using the same CM7/1 cell line, demonstrated that 2.1×10^6 cardiomyocytes could be generated from 1×10^6 input ES cells (C/ES = 2.1) on rotating suspension cultures (Zweigerdt et al., 2003). In the current study, experiments were performed using bulk transfected D3 ES cells, providing evidence that our results were reproducible in other cell lines. A summary of the improvements to cardiomyogenic yield

Figure 6. Improved cardiomyocyte yield per input ES cell was observed under hypoxic conditions. **A:** Oxygen tension profiles in controlled hypoxic and normoxic bioreactor cultures. **B:** Cell density in hypoxia normalized to cell density in normoxia in the controlled bioprocess. **C:** Representative flow cytometry plots for MF20 analyses of cells from the hypoxic and normoxic bioreactors on d14. **D:** Cardiomyocyte yield per input ES cell on d14.

enabled by encapsulation and hypoxic culture are outlined in Table I. We report here a C/ES of 0.16 for differentiation of D3 cells using the S/SF culture system, significantly lower than that reported for the CM7/1 cell line (Zandstra et al., 2003). Despite the difference in the magnitude of cardiomyocyte yield achieved, likely due to variability between cell lines (cell growth, cardiomyogenic potential, etc.), the general trends (cell density and cardiomyocyte frequency with respect to time) of the system remain similar between these cell lines. Furthermore, in the bioprocess described here we achieved a C/ES of 3.77, higher than previously reported for any other differentiation system or cell line.

An important aspect of the bioprocess design required implementing a method to prevent EB agglomeration, as this phenomenon inhibits efficient cell growth, thus resulting in lower cardiomyocyte yield. Encapsulation is a technique that provides a barrier between EBs, thereby blocking contact between e-cadherins, adhesion-promoting surface molecules expressed by ES cells, from separate EBs (Dang et al., 2004). Encapsulation prevented EB agglomeration in stirred suspension and contributed to significantly enhanced cardiomyocyte yields. Moreover, encapsulation permits direct differentiation in stirred suspension, thereby allowing us to monitor and control culture conditions from the start of differentiation, and minimizes handling and disturbance of the cultures during early differentiation. Encapsulation will contribute to further development of this bioprocess, specifically for scale-up, as similar cardiomyocyte yields were achieved in encapsulated cultures with 24 times fewer input ES cells.

As we have shown, in perfusion culture systems supplementation is controlled, metabolic wastes do not accumulate, and the severe dilution of cell-secreted factors that occurs in semicontinuous medium exchanges is reduced. However, perfusion may offer additional benefits as this process is further developed. It has been reported that blood stem/progenitor cell growth is enhanced in perfusion controlled bioreactors, likely due to the continuous removal of cell differentiation inducers and metabolites (Koller et al., 1993a,b; Van Zant et al., 1994). It has also been demonstrated in human bone marrow perfusion bioreactors that the types of cells produced can be affected by the consumption and production of a variety of growth factors in the medium (Koller et al., 1995), and that endogenous growth factor production may be stimulated by increased medium exchange (Caldwell et al., 1991). Thus, in future development of the bioprocess, perfusion may be involved in improving cell expansion as well as improving cardiomyocyte production specifically. Ongoing studies are taking advantage of the perfusion control system to reduce the cell density fluctuations (due to initial cell growth followed by significant nontarget cell death upon selection) by employing methods such as metabolic activity-based feeding.

During embryogenesis, and particularly the development of the cardiovascular system, many of the developmental processes that occur involve hypoxia, as diffusion of oxygen becomes limited by the growth of the embryo (Ramirez-Bergeron and Simon, 2001). We observed that hypoxia had a beneficial effect on ES cell-derived cardiomyocyte yield. Hypoxia activates the expression of hypoxia inducible factor 1 (HIF-1), which may indirectly enhance cardiomyocyte differentiation by the activation of a number of growth factors, including vascular endothelial growth factor (VEGF), erythropoietin (EPO), and basic fibroblast growth factor (bFGF) (Ramirez-Bergeron and Simon, 2001), which have a synergistic effect on mesoderm differentiation processes, including cardiogenesis (Ramirez-Bergeron and Simon, 2001; Semenza, 2001). FGF-4 and VEGF have each been shown to enhance cardiac differentiation in combination with bone morphogenetic protein (BMP-2 or -4) (Lopez-Sanchez et al., 2002; Lough et al., 1996; Nakayama et al., 2000; Peng et al., 2002). BMPs are secreted by the anterior endoderm during embryogenesis and have been shown to promote cardiomyocyte generation (Sachinidis et al., 2002). VEGF may work by enhancing cell survival of BMP-induced mesoderm formation, and thus affect the development of cardiomyocytes. Our system allows, for the first time, the investigation of these mechanisms, by directly measuring and controlling oxygen in differentiating ES cell cultures.

Ultimately, development of this bioprocess leads towards studies utilizing human ES cells (hESC). The mouse system provides a preliminary understanding of the effects of different bioprocess parameters on differentiation to cardiomyocytes. Several differences have already been observed for hESC cardiomyogenic development, some of which may impact bioprocess-related parameters. For instance, beating frequency in differentiating hESC appears to be less frequent than in mouse ES cells (Kehat et al., 2001), suggesting that a lower number of cardiomyocytes may be produced during human EB growth. It has been observed that cardiac differentiation in hESC can be maintained for 260 population doublings ($\sim$50 passages), whereas late-passage mES cells may have difficulty differentiating to cardiomyocytes (Xu et al., 2002). Finally, enhancers of cardiomyocyte differentiation for mES cells, such as dimethyl sulfoxide (DMSO) and retinoic acid (RA), do not appear to have the same effect on hESC (Xu et al., 2002). Current investigations are adapting the bioprocess to human cells.

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